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(71) Applicants: THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, U.S. DEPARTMENT OF COMMERCE [US/US]; 5285 Port Royal Road, Springfield, VA 22161 (US). CELLCO, INC. [US/US]; 12321 Middlebrook Road, Germantown, MD 20874 (US).

(72) Inventors: CULVER, Kenneth, W. ; 5 Enid Court, Potomac, MD 20854 (US). KNAZEK, Richard, A. ; 10922 Brewer House Road, Rockville, MD 20852 (US). BLAESE, R., Michael ; 1986 Lancashire, Rockville, MD 20854 (US).

(74) Agents: STERN, Marvin, R. et al.; Fleit, Jacobson, Cohn, Price, Holman & Stern, The Jenifer Building, 400 Seventh Street, N.W., Washington, DC 20004 (US).

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(54) Title: SUSTAINED AND CONTINUOUS PRODUCTION OF HIGH TITERS OF RECOMBINANT VIRAL VECTORS AND TRANSDUCED TARGET CELLS FOR USE IN GENE THERAPY

(57) Abstract

   This invention provides improved methods for producing high titers of recombinant viral vectors by culturing producer cells in a hollow fiber bioreactor. The titer of virus in the extra fiber space (EFS) of the hollow fiber bioreactor is sufficient to transduce target cells at a multiplicity of infection sufficient to render the transduced target cells useful for genetic therapy. In a preferred embodiment, target cells are transduced with the EFS medium from a hollow fiber bioreactor in which a producer cell line, which releases packaged retroviral vectors into the EFS, has been cultured. Lymphocytes derived from an adenosine deaminase (ADA)-deficient individual that have been transduced with EFS medium that contains retroviral vectors that include RNA that encodes ADA, express ADA at a rate comparable to that of cells from an individual who does not have ADA deficiency.

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SUSTAINED AND CONTINUOUS PRODUCTION OF HIGH TITERS
OF RECOMBINANT VIRAL VECTORS AND TRANSDUCED
TARGET CELLS FOR USE IN GENE THERAPY

FIELD OF INVENTION

5   This invention is directed to methods for
producing high titers of viral vectors in vitro in a
hollow fiber bioreactor, to methods for infecting target
cells at high multiplicity and for producing high
concentrations of transduced target cells, and to the

10   transduced target cells produced by the methods. The
methods are particularly suited for producing transduced
target cells for use in methods of genetic therapy.

BACKGROUND OF THE INVENTION

Genetic therapy.

15   Genetic therapy for treatment of acquired and
inherited diseases is a recent and highly promising
addition to the repertoire of treatments for such
diseases. It is expected that many congenital genetic
abnormalities and acquired diseases will be amenable to

20   treatment by genetic therapy. Genetic therapy can be
effected by removing selected cells, target cells, from
an afflicted individual, modifying the cells by
introducing heterologous DNA that encodes a
therapeutically effective product and returning the

25   modified cells to the individual. Eventually it may be
possible to introduce the heterologous DNA directly into
cells in vivo, such as endothelial cells that line the
lungs, without any in vitro manipulation of the target
cells.

30   Diseases that are candidates for such treatment
include those that are caused by a missing or defective
gene that normally encodes an enzyme, hormone, or other
protein. Examples of such diseases include: a severe
combined immunodeficiency disorder, which is caused by a

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defect in the DNA that encodes adenosine deaminase (ADA)
(see, e.g., Kredich et al. (1983), p. 1157, in The
Metabolic Basis of Inherited Disease (5th ed.), eds.
Stanbury, et al., McGraw-Hill, New York); Lesch Nyhan
5   disease, which is caused by a defect in the enzyme
hypoxanthine-guanine phosphoribosyl transferase (HGPRT);
cystic fibrosis and Duchenne muscular dystrophy for which
the respective defective genes have recently been
identified; Tay sachs disease; and hemoglobin disorders,
10   such as β-thalassemia. In addition, genetic therapy has
been proposed as a means to deliver therapeutic products,
such as tumor necrosis factor (TNF) for the treatment
cancers and CD4 receptor protein for the treatment of
AIDS (see, e.g., PCT International Application No. WO
15   90/01870).

Genetic therapy involves introducing
heterologous DNA into at least some cells of a host
organism in a manner such that the products encoded by
the heterologous DNA are expressed in the host. Upon
20   introduction into the host cell, the heterologous DNA may
be integrated into the genome of the host cells or it may
be maintained and replicated as part of an episomal
element. The heterologous DNA may encode products that
replace or supplement the product of a defective or
25   absent gene or a gene that is normally expressed at low
levels or the DNA may encode therapeutic products that
are effective for treating a disease. The heterologous
DNA is operatively linked to a promoter and/or other
transcriptional and translational regulatory elements
30   that are recognized by host cell effector molecules, such
as RNA polymerase II, such that it can be expressed in
the host cell. As understanding of the underlying
genetic bases for disease increases, it will be possible

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to refine the methods of genetic therapy so that regulatory controls that operate at the level of gene transcription or translation or that rely on mechanisms, such as feedback inhibition, to control expression of
5   gene products can also be provided to the host cells. For example, the heterologous DNA may also mediate or encode RNA or protein products that mediate expression of a host cell gene or biochemical process. Expression of the heterologous DNA can thereby be fine-tuned to the
10 needs of the afflicted host.

It is also anticipated that numerous means for introducing heterologous DNA into the cells and genomes of individuals will be developed and refined. At the present time, the use of recombinant viral vectors, which
15 are derived from viruses that infect eukaryotic cells, provide the most promising means for effecting genetic therapy. Generally, upon infection of a eukaryotic host, a virus commandeers the transcriptional and translational machinery of the host cell. In order to do so, viral
20 regulatory signals, such as promoters, particularly those recognized early in infection, tend to be highly efficient so that any DNA that is in operative linkage with such promoters and regulatory signals is efficiently expressed at high levels. Eukaryotic viruses have,
25 therefore, been used as vectors for cloning and expression of heterologous DNA in eukaryotic cells.

Recombinant eukaryotic viruses for delivery of heterologous DNA.

Eukaryotic viruses from which recombinant viral
30 vectors have been constructed include both DNA viruses, such as SV40, adenovirus, and bovine papilloma virus (see, _e.g._, Gluzman, Y., ed. _Eukaryotic Viral Vectors_, Cold Spring Harbor Laboratory, Cold Spring

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Harbor, NY (1982); Sarver _et al._ (1981) <u>Mol. Cell Biol.</u>
1: 486; and U.S. Patent No. 4,419,446 to Howley), and
RNA viruses, retroviruses, such as Moloney murine
leukemia virus (MoMLV), mouse mammary tumor virus (MMTV),
5 Rous sarcoma virus (RSV) and other leukemia and tumor
viruses (see, _e.g._, Mann _et al._ (1983) <u>Cell</u> 33: 153-
159; Miller _et al._ (1986) <u>Mol. Cell. Biol.</u> 6: 2895-
2902; U.S. Patent No. 4,868,116 to Morgan _et al._; U.S.
Patent No. 4,686,098 to Kopchick _et al._; and U.S. Patent
10 No. 4,861,719 to Miller).

**Design of retroviral vectors for use in methods of
genetic therapy.**

      Retroviral vectors are presently the preferred
vectors for genetic therapy (see, _e.g._, Anderson (1984)
15 <u>Science</u> 226: 401-409) because retroviral infection is
highly efficient and retroviral vectors can be readily
modified so that heterologous DNA carried by such vectors
is stably integrated into the host cell genome. If
retroviral vectors could be produced at a sufficiently
20 high concentration, virtually 100% of exposed target
cells, cells that are derived from the afflicted host,
could be infected and express integrated proviral and
heterologous DNA. Upon infection with a retrovirus, and
under appropriate conditions, a single copy of a provirus
25 integrates per cell. Proviral integration is not, per
se, harmful to the cell. Also, because of the size and
mechanism of retroviral integration, it is possible to
know precisely what DNA has been integrated. Finally,
retroviral vector systems that have a broad host range
30 are readily available.

      Retroviruses consist primarily of a protein
envelope that encapsulates core proteins and RNA. The
RNA of a retrovirus encodes two long terminal repeat

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sequence (LTRs), which include promoter and enhancer regions and which flank the genome; various regulatory signals that regulate transcription, including the CAP site and polyadenylation signals, and that regulate
5   reverse trans-cription and proviral replication; structural genes inclu-ding the _env_ gene, which encodes the envelope proteins, the _gag_ gene, which encodes viral core proteins, and the _pol_ gene, which encodes the reverse transcriptase. The retro-viral RNA also includes
10   signal sequences, such as the tRNA binding site (the replication initiation site for minus DNA strand synthesis), the replication site for plus DNA strand synthesis, and the packaging signal, the psi site.

Retroviral envelope proteins include regions
15   that recognize and specifically bind to mammalian cell surface receptors. Some retroviral envelope proteins only bind to a restricted range of host cells; viruses encapsulated in such envelopes are said to have an ecotropic host range. Other envelope proteins bind to a
20   variety of mammalian cells; viruses encapsulated in such envelopes are said to have an amphotropic host range. Upon specific recognition and binding to host cell receptors, the virus enters the cell. The retroviral reverse transcriptase is translated and the virus is
25   reverse transcribed into a DNA intermediate, referred to as a provirus, which integrates into chromosomal DNA. Proviral DNA can also be replicated and packaged into infectious virions.

Elements for retroviral replication are divided
30   into those that act in _cis_ and those that act in _trans_. _Trans_-acting factors include the viral proteins that are necessary for encapsidation, binding and entry of the virus into a target cell, reverse transcription, and

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integration of the reverse transcribed DNA into the target cell genome. _Cis_-acting factors, such as the packaging signal, include those that interact with the _trans_-acting proteins and other proteins during viral replication (see, _e.g._, Coffin, J. (1985) in _RNA Tumor Viruses_, vol. 2, pp. 17-74, R. Weiss _et al._, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Some of the _cis_- and _trans_-acting functions can be deleted from a retrovirus and, if properly combined, provided separately. A virus that has some or all of the _trans_-acting functions deleted is replication-incompetent, but, if missing functions are provided, such as by co-transfection with a helper virus containing the necessary functions, packaged defective infectious viral particles can be produced. Alternatively, missing functions can be provided by a cell line, a packaging cell line, that has been modified by stable incorporation of such functions in its genome. Because certain functions can be deleted and provided by way of a helper virus or as part of a packaging cell line, retroviral vectors for delivering heterologous DNA, which is then stably integrated into host cell DNA can be constructed. In addition, by careful design of packaging cell lines and retroviral vectors, it is possible to package infectious replication incompetent retroviral vectors without producing helper virus and thereby provide vectors for integrating DNA into a host cell genome without the concomitant risk of recombinational events between the vector and helper virus that could lead to the production of infectious retroviral particles.

Retroviral vectors are constructed by preparing DNA copies of the retroviral RNA and deleting all or parts of the _env_, _pol_ and _gag_ genes. Heterologous DNA is

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inserted in place of the deleted genes under the control
of the endogenous heterologous promoter or other promoter
recognized by a host cell RNA polymerase II, or the
retroviral 5' LTR.   Retroviral vectors, thus, do not

5   include genes for replication. When they are transduced,
in the absence of helper virus, into cells that do not
include such retroviral sequences, the retroviral vectors
cannot replicate and, if properly constructed, they are
stably incorporated into the host cell genome.   In

10 addition to the LTR sequences and other <u>cis</u>-acting
regulatory sequences, retroviral vectors generally also
include splice donor and acceptor sites and a selective
marker gene, such as the bacterial gene, <u>neo</u>, which
encodes neomycin phosphotransferase, which confers

15 resistance to certain antibiotics, under the control of
an appropriate eukaryotic promoter.

The host range of the packaged retroviral
vector can be controlled by selection of the <u>env</u> gene
that is incorporated into the packaging cell line. If an

20 amphotropic host range is desired, then the vector is
packaged in a packaging cell line that includes <u>env</u>
sequences derived from a retrovirus that has an
amphotropic host range.   For example, the MoMLV (see,
<u>e.g.</u>, Mann <u>et al.</u> (1983) <u>Cell</u> 33:   153-159; and Miller

25 <u>et al.</u> (1986) <u>Mol. Cell. Biol.</u> 6: 2895-2902) is an
amphotropic retrovirus;   its <u>env</u> protein binds to
receptors present on most human cells.

Since retroviral vectors do not replicate in the
target host cell, retroviral vectors are replicated and

30 packaged in cell lines that include DNA that encodes
functions, absent in the vector, that are necessary for
packaging and replication.   Because of the ease with
which retroviruses integrate and excise from chromosomal

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DNA and undergo recombination, recombination between the
DNA derived from the vector and DNA in the packaging cell
line may result in production of packaged, replication
competent viruses and/or helper viruses, which encode
5   functions necessary for viral replication.   Upon
transduction into a target host cell, in the presence of
helper viruses, recombination can result in the
production of infectious retroviral particles in the host
cells.   Consequently, for clinical use, not only must
10 retroviral vectors be replication incompetent, the
packaging cell line and vector must be designed so that
there is virtually no possibility of recombination that
could lead to the production of replication competent or
helper viruses. This is achieved by carefully designing
15 both the vector and the packaging cell line to include
deletions and mutations that would render it highly
improbable   or   impossible   for   any   undesirable
recombinational events between the retroviral vector and
packaging cell line (see, e.g., Mann et al. (1983) Cell
20 33: 153-159; Miller et al. (1986) Mol. Cell. Biol. 6:
2895-2902 and (1985) Mol. Cell. Biol. 5: 431; and U.S.
Patent No. 4,861,719 to Miller). There is, however, the
minute possibility of undesirable recombinational events
between the vector and sequences carried on the host cell
25 genome that could result in the production of helper
virus or activation of cellular oncogenes. These risks
are, however, minute and retroviral vectors have been
designed that render the probability of such occurrences
insignificant.

30 **Design of packaging cell lines for production of
clinically useful recombinant retroviral vectors.**

Manipulation of the viral genome has, thus,
permitted construction of retrovirus-packaging cell lines

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that can produce relatively large amounts of viral vectors in the absence of both replication-competent virus and helper virus. These cell lines package retroviral vector RNA into virions that are capable of
5   infecting a broad range of target host cells, but, that, after infection of such cells, cannot replicate. Packaging cell lines contain retrovirus-derived DNA that supplies the necessary gene functions, such as the env gene, for viral packaging. Most such packaging cell
10 lines contain helper virus DNA that has been modified by deletion of the packaging signal. It has been found, however, that packaging cell lines in which only the packaging signal is deleted will produce helper virus at low frequency and also interact with some retroviral
15 vectors to yield replication-competent virus at low levels. Thus, additional mutations are introduced into the retroviral DNA in the packaging cell lines in order to further decrease probability for production of helper virus and/or also replication-competent virus (see, e.g.,
20 Miller et al. (1986) Mol. Cell. Biol. 6: 2895-2902 and U.S. Patent No. 4,861,719 to Miller).

Packaging cell lines are derived from transformed or immortalized cell lines, such as NIH 3T3 cells, and particularly from NIH 3T3 thymidine kinase (TK⁻
25) cells. A DNA construct, such as a plasmid, containing the retroviral sequences with the desired deletions and mutations and a selective marker, such as the herpes simplex virus (HSV) thymidine kinase (TK) gene are introduced into the cell line, such as the NIH 3T3 TK⁻
30 cells, and cultured in selective medium. Cells, which grow in the selective medium, are selected and tested for the presence of the necessary packaging functions. Those that produce retroviral vectors and do not produce helper

viral are selected and used as packaging cell lines to produce infectious replication-incompetent retroviral vectors.

Target cells and their use in genetic therapy.

5   Suitable target cells for gene transfer are those that readily can be obtained and that persist following transplantation, such as fibroblasts, immune cells, particularly lymphocytes, and epithelial cells (See, _e.g_, St. Louis _et al._ (1988) Proc. Natl. Acad. Sci.

10 85: 3150-54; Keller _et al._ (1985) Nature 318: 149-154; Miller _et al._ (1988) J. Virol. 62: 4337-4345; and Morgan _et al._ (1989) U.S. Patent No. 4,868,116).

The first use of genetic therapy in humans involved tumor infiltrating lymphocytes (TILs) as target

15 cells (see, Rosenberg _et al._ (1990) New Engl. J. Med. 9: 570-578). TILs are a lymphocyte subpopulation that show promise as vehicles for delivery of anti-cancer therapeutics to tumor sites. These lymphocytes infiltrate into tumors, as part of an attempt by the

20 host's immune system to mount an immunological response.

TIL cells for use as target cells for genetic therapy can be produced _in vitro_ by incubating resected human tumors, such as kidney, colon or breast tumors, melanomas, and sarcomas _in vitro_ in appropriate tissue

25 culture medium that contains interleukin-2 (IL-2). The IL-2 in the medium results in the expansion and activation of T cells within the tumor, the TIL cells, and the destruction of tumor cells or tissue. After 2-3 weeks in culture, the tumor cells have been destroyed and

30 the culture primarily contains lymphoid cells that have the phenotype of cytolytic T lymphocytes (CTL) (see, _e.g._, Rosenberg _et al._ (1988) N. Engl. J. Med. 319:1676-1680; Muul _et al._ (1987) J. Immunol. 138: 989-995; and

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Topalian et al., (1989) J. Immunol. 142: 3714-3725).

TIL cells also show promise for use in methods
of genetic therapy, particularly cancer therapy, (see,
e.g. Culliton (1989), "News and Comment" in Science 244:
5   1430-1433 and Kasid et al. (1990) Proc. Nat'l. Acad. Sci.
87: 473-477) because they provide a source of autologous
cells that target tumors and that can be modified by the
insertions of DNA encoding a desired protein, cultured,
and reintroduced into the patient.   Recently, TILs
10 containing DNA encoding a bacterial marker gene, neo,
which encodes neomycin phosphotransferase, were infused
into the veins of melanoma patients in order to track the
fate of the TILs after infusion in the patients (see
Rosenberg et al. (1990) New Engl. J. Med. 9: 570-578).
15 The   gene was inserted into a retroviral vector, which
was then introduced into a retroviral packaging cell line
(Miller et al. (1986) Mol. Cell Biol. 6:   2895-2902).
The packaging cell line was cultured and yielded packaged
defective virions at titers sufficient to transduce TIL
20 cells at a multiplicity of infection of virions to cells
of about 1.3 to 2.3. After transduction, the cells were
cultured overnight, and, in an effort to increase the
number of cells infected, the TILs were again exposed to
the virus.   Although   only 1 to 11% of the cells were
25 transduced, it was possible to locate and identify the
infused TIL in the treated patients for at least 64 days.

TILs from patients with advanced melanoma have
been modified by insertion of DNA encoding TNF and will
be reinfused into the patients in an effort to enhance
30 the anti-tumor activity of the TIL cells.

The first experiment in genetic therapy for the
treatment of a genetic disorder, a severe combined
immunodeficiency disease, is presently underway.   ADA

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deficiency, which is associated with a severe combined immunodeficiency disease, is a fatal condition because the ADA substrates, deoxyadenosine and adenosine, which are toxic to T and B lymphocytes, accumulate in serum of

5   the affected individual.   In the hope of treating this disorder, a retroviral vector containing DNA encoding adenosine deaminase (hereinafter ADA) (Hock et al. (1989) Blood 74: 876-881) has been introduced into lymphocytes obtained from a child ADA deficiency.

10   The retroviral vector was packaged in vitro using a cell line shown to produce relatively high titers of the retrovirus containing the ADA gene without concomitant production of helper virus.   Although the titers produced by the packaging cells were relatively

15 high, they were only high enough to infect at a multiplicity of infection of about 1 virion/target lymphocyte, which was sufficient to transduce at most about 10% of exposed target cells after repeated exposures of the target cells to the packaged retroviral

20 vectors.   The transduced lymphocytes were then infused into the child.   It is hoped that after repeated infusions of similarly transduced lymphocytes that sufficient levels of ADA will be expressed to reduce the concentrations of toxic metabolites and thereby permit

25 development of a normal array of immune cells.

            Since lymphocytes have a limited lifespan, infusions of transduced lymphocytes will have to be repeated at regular intervals.   For each such infusion lymphocytes will have to be transduced and each

30 transduction will require multiple exposures of the lymphocytes to the packaged retroviral vectors because only relatively low titers of the retroviral vectors can be obtained.   Even with as many as six exposures of the

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lymphocytes to packaged retroviral vectors, only about 10% of the cultured lymphocytes will be transduced. This procedure is, therefore, costly and, unless improved, will not be available for general clinical use.

5   Because retroviral particles are fragile, they cannot be concentrated by any means known to those of skill in the art. The concentration (transduced cells/total number of target cells x 100) and total number of transduced target cells that can be obtained is
10   limited by the titer of the retroviral particles produced by the packaging cell line, which in turn is limited by the concentration of packaged particles that are released into the culture medium bathing the packaging cell line. Generally, only titers of from 7 x 10^3 to 5 X 10^5 CFU/ml
15   can be obtained (see, e.g., U.S. Patent No. 4,861,719 to Miller), which severely limits their usefulness in genetic therapy. In order to transduce sufficient numbers of target cells, however, it is necessary to have titers of at least 10^6 to 10^7 CFU/ml) (Miller et al.
20   (1988) J. Virol. 62: 4337-4345)

It is, therefore, an object of this invention to provide a method for producing recombinant viral vectors, particularly recombinant retroviral vectors, at high titers.

25   It is another object of this invention to provide methods for transducing target cells at a high multiplicity of infection.

It is another object of this invention to provide a dual bioreactor system and methods for
30   efficiently trans-ducing target cells at high multiplicities of infection.

It is another object of this invention to provide high concentrations of transduced target cells

that can be used in methods of genetic therapy.

SUMMARY OF THE INVENTION

Methods for producing of titers of recombinant viral vectors by culturing producer cells in a hollow fiber bioreactor are provided. In particular, methods for producing sustained and continuous production of a high titer of recombinant eukaryotic viral vectors, particularly recombinant retroviral vectors, by culturing a packaging cell line in a hollow fiber bioreactor are provided.

High titers of recombinant retroviral vectors are secreted into the overlying medium, the extra fiber space (hereinafter EFS) medium by producer cells, packaging cells, that are cultured in a hollow fiber bioreactor are also provided.

The ability to produce high titers of recombinant viral vectors permits transduction of sufficient concentrations of target cells to be useful for genetic therapy. Target cells are transduced by contacting the target cells with the EFS medium from the bioreactor in which the producer cells are cultured.

In a preferred embodiment a packaging cell line containg DNA that is derived from a retroviral vector is cultured in the bioreactor. High titers, generally at least about 10^6 CFU/ ml, of infectious packaged retroviral vectors accumulate in the EFS medium. If the EFS medium is harvested and replaced with fresh medium, the producer cells continue to secrete viral vectors at a high rate, generally at least about 10^8 retroviral particles/ml per day. The EFS medium can be repeatedly harvested and production of recombinant retroviral vectors continues and is sustained at the high level.

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The EFS medium that contains the recombinant retroviral vectors is contacted with selected target cells, particularly lymphocytes, at a multiplicity of infection that may be as high as about 10 CFU/target

5   cell. The target cells may then be cultured in a second bioreactor. The target cells may be contacted with multiple EFS media harvests from the packaging cell line. It should be possible to transduce up to 100% of the target cells.

10   In a preferred embodiment, a dual bioreactor system in which the EFS from a first bioreactor in which the producer cells are cultured is connected with the EFS of a second bioreactor in which the target cells are cultured. This assembly efficiently and continuously

15 transduces the target cells by introducing the medium in the EFS from the first bioreactor into the EFS of the second bioreactor. Means for collecting spent EFS in the second bioreactor and adding fresh medium to the EFS of the first bioreactor are included in the system.

20   Examples are provided that demonstrate that the EFS medium obtained from a hollow fiber culture of a packaging cell line produces high titers of the retroviral vector LASN, which contains nucleic acid that encodes ADA. The packaged LASN retroviral vectors have

25 been used to efficiently transduce ADA-deficient lymphoid cells, which subsequently expressed ADA activity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a presents titer (vector particles/ml) as a function of duration of culture in the bioreactor.

30 After 50 days in culture the titer of viral vector particles plateaued at more than 10^6 particles/ml.

Figure 1b presents the same data expressed as packaged retroviral vector particle production per day as

a function of time in culture.   Vector particle
production per day increases substantially with duration
of the culture.

        Figure 2 presents a schematic diagram of a dual
5   perfusion circuit for direct and continuous inoculation
of the EFS from a hollow fiber bioreactor that contains
a viral packaging or producing cell line into the EFS of
a hollow fiber bioreactor that contains target cells.
The   perfusion   circuit   includes: reservoirs   (a),
10 connecting tubing (b), media pumps (c), and hollow fiber
bioreactors (d).   Cells are injected into the EFS
through the loading side-ports (f).

        Medium from the EFS of the virus-producing
hollow fiber bioreactor is pumped either continuously,
15 periodi-cally or intermittently into the EFS of the
target cell hollow fiber bioreactor.   The circuit also
includes   reservoir   bottles   that   contain   the   EFS
replacement medium (1) for the virus-producing bioreactor
and for collecting the spent EFS medium (2) from the
20 target cell bioreactor Several smaller reservoir bottles
(4) are included in the connecting lines between the EFS
of the two bioreactors and between the reservoirs (1) and
(2).   The   smaller   bottles   may   be   used   for   sample
withdrawal, inoculation, or for displacement of the EFS
25 medium in either bioreactor with sterile air.   There are
filters (f) on the each of the reservoirs and smaller
bottles.   Clamps (X) are present in the various lines to
direct the flow of medium.   Connectors (C_o), which may be
placed in any of the lines, permit removal and
30 replacement of any component of the circuit.   An
automatic pinch valve, which opens when the peristaltic
pump (P) is activated and closes when the pump is not
pumping, may also be included in the circuit.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which
5 this invention belongs.   All publications mentioned herein are incorporated by reference thereto. All U.S. patents mentioned herein are incorporated in their entirety by reference thereto.

Definitions

10   As used herein, genetic therapy involves the transfer of heterologous DNA to the certain cells, target cells, of an individual afflicted with a disorder for which such therapy is sought. The DNA is introduced into the selected target cells in a manner such that the
15 heterologous DNA is expressed and a product encoded thereby is produced. Alternatively, the heterologous DNA may in some manner mediate expression of DNA that encodes the therapeutic product, it may encode a product, such as a peptide or RNA that in some manner mediates, directly
20 or indirectly, expression of a therapeutic product. Genetic therapy may also be used to introduce therapeutic compounds, such as TNF, that are not normally produced in the host or that are not produced in therapeutically effective amounts or at a therapeutically useful time.
25 The heterologous DNA encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

As used herein, heterologous DNA is DNA that
30 encodes RNA and proteins that are not normally produced in vivo by the cell in which it is expressed or that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation,

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or other regulatable biochemical processes. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed

5   is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer

10   agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

15   As used herein, a therapeutically effective product is a product that is encoded by heterologous DNA that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired

20   disease or that cures said disease.

          Typically, DNA encoding the desired heterologous DNA is cloned into a plasmid vector and introduced by routine methods, such as calcium-phosphate mediated DNA uptake (see, (1981) Somat. Cell. Mol. Genet. 7:603-616)

25   or microinjection, into producer cells, such as packaging cells. After amplification in producer cells, the vectors that contain the heterologous DNA are introduced into selected target cells.

          As used herein, operative linkage of

30   heterologous DNA regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences refers to the relationship between such

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DNA and such sequences of nucleotides.   For example,
operative linkage of heterologous DNA to a promoter means
that the DNA and the promoter are spatially related such
that the transcription of such DNA is initiated from such
5   promoter by an RNA polymerase that specifically
recognizes, binds to the promoter and transcribes such
DNA.   Heterologous DNA may be introduced into a cell by
any method known to those of skill in the art.

As used herein, a target cell is a cell into
10   which heterologous DNA is introduced for expression in
the host who is being treated.   Such heterologous DNA may
encode a gene product, such as an enzyme, that certain
individuals do not express or express in a form that is
defective.   Suitable target cells are known to those of
15   skill in the art and include, but are not limited, to
fibroblasts (see, e.g., St. Louis et al. (1988) Proc.
Natl. Acad. Sci. 85: 3150-54) and immune cells (see,
e.g., Keller et al. (1985) Nature 318:   149-154 and
Miller et al. (1988) J. Virol. 62: 4337-4345).   Target
20   cells may be removed from the individual who is being
treated and modified by introducing the heterologous DNA
in vitro.   For example, target cells, such as
lymphocytes may be transduced with retroviral vectors
that have been produced by producer cells.
25   Alternatively, it may be possible to modify target cells,
such as endothelial cells that line the lungs, in vivo.

As used herein, transduced target cells refers
to the portion of target cells that, after contacting
target cells with a recombinant vector that includes a
30   heterologous DNA, contain the heterologous DNA or contain
the heterologous DNA and express the product of the
encoded by the heterologous DNA.   Genetically engineered
target cells, target cells that contain heterologous DNA,

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are used in genetic therapy to correct genetic disorders, such as, but not limited to, certain immunodeficiency diseases, β-thalassemia, Gaucher's disease, hemophilia and cystic fibrosis, by introducing

5   them into an individual who has an inherited or acquired genetic defect. In addition, target cells may be genetically engineered to also express DNA encoding drug resistance, such as methotrexate resistance, or drug sensitivity, such that, when such DNA is expressed, the

10   cells may be selectively expanded or destroyed _in vivo_ and to express thereapeutically effective substances, including antibodies and tumor necrosis factor.

As used herein, transduction is the process whereby a viral vector specifically binds to cell surface

15   receptors and enters the cell. It is a process akin to viral infection, except that viral vectors are modified viruses and, upon introduction, into a target cell, generally, do not cause productive infection. For example, retroviral vectors are generally designed to be

20   replication-incompetent.

As used herein, the concentration of transduced target cells refers to the number of transduced target cells/total number of target cells contacted with the vector. The concentration may be expressed as a

25   percentage (number of transduced target cells/total number of target cells x 100).

As used herein, a recombinant viral vector is a vector that includes DNA that is derived from an RNA or DNA virus and also includes heterologous DNA, which is

30   generally in operative linkage with a promoter and other transcriptional and translational regulatory sequences or signals that are recognized by the host cell in which the virus from which such vector is derived can replicate.

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Recombinant vectors may be either retained as part of independently replicating as episomal elements or integrated into the genome of the host cell.

Recombinant viral vectors useful for genetic therapy are typically derived from viruses that infect and replicate in eukaryotic cells and thereby serve as a means for introducing heterologous DNA into eukaryotic cells. Recombinant viral vectors that remain episomal include an origin of replication, whereby DNA synthesis can be initiated. Recombinant vectors that integrate into the genome must include DNA sequences necessary to effect integration. Preferred recombinant viral vectors used for genetic therapy are generally selected from among those which integrate into the host cell genome.

As used herein, a producer cell is cell in which recombinant viruses can replicate and can thereby be amplified. Some producer cells also package and secrete recombinant viruses into the medium in which the cells are cultured. The recombinant viral vectors produced by producer cells are used to transduce target cells. Producer cells are typically immortalized or transformed cell lines that are cultured _in vitro_ and are designed to produce maximal amounts of recombinant vectors. For example, retrovirus packaging cell lines are producer cells that include trans-acting factors necessary to package defective retroviral vectors.

As used herein, adoptive immunotherapy is a therapeutic method, whereby cells of the immune system are removed from an individual, cultured and/or manipulated _in vitro_, and introduced into the same or a different individual as part of a therapeutic treatment for an acquired or inherited disease. Immune cells and adoptive immunotherapeutic methods may be adapted for use

in methods of genetic therapy as vehicles for delivery of
heterologous DNA.

As used herein, immune cells include any cells
that participate the functioning of the immune system.
5   Lymphoid cells include lymphocytes, macrophages, and
monocytes that are derived from any tissue in which such
cells are present. In general lymphoid cells are removed
from an individual who is to be treated.

As used herein, a growth promoting substance is
10   a substance, that may be soluble or insoluble, that
in some manner participates in, induces cells or
otherwise activates cells, directly or indirectly, to
differentiate or proliferate. Growth promoting
substances include mitogens and cytokines, including
15   interleukins, colony stimulating factors, and any other
of such factors that are known to those of skill in the
art. For example, many types of cells, including target
cells, such as lymphocytes, which in vitro require IL-2,
have absolute requirements for certain growth promoting
20   substances. Growth promoting substances are well known
to those of skill in the art. Many such substances, such
as the interleukins 1-7, have been cloned and expressed
in vitro. It is within the level of skill in the the
art, to select appropriate growth factors in order for
25   culturing both producer cells and target cells in a
bioreactor. Other substances, including polycations,
such as protamine, may be added to the bioreactor in
order to promote viral infectivity by, for example,
enhancing viral adsorption to the target cell surface.

30   As used herein, a therapeutically effective
amount of transduced target cells is a sufficient
concentration and number of transduced target cells for
at least single infusion of such cells into an individual

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for genetic therapy. Upon infusion of a therapeutically effective amount of transduced target cells, a sufficient amount of a product produced by the transduced cells is expressed to ameliorate or eliminate the symptoms or

5   manifestations of an inherited or acquired disease. In order to effect a cure or a substantial reduction of the symptoms or manifestations of the disease, it may be necessary to repeat perform multiple infusions and/or to periodically repeat such infusions.

10   As used herein, a hollow fiber culture system consists of a hollow fiber bioreactor and means for pumping and collecting perfusing medium. A hollow fiber bioreactor is a hollow shell that encases a plurality of semi-permeable fibers through which medium is perfused.

15   As used herein, the extra fiber space (EFS) is the space in which the cells grow that is external to the semi-permeable fibers and bounded without by the shell of the hollow fiber bioreactor. The EFS is alternatively referred to as the extra capillary space (ECS).

20   As used herein, the EFS cell medium is the medium in which the cells in the EFS are growing. It may also be referred to as the EFS supernatant. It contains secreted cellular products, including secreted viral particles, diffusible nutrients and any other compounds,

25   including growth promoting or suppressing substances, such as lymphokines and cytokines, that have been added to the EFS medium, or diffusible products that have been added to the perfusing tissue culture medium. The particular components included in the EFS is a function

30   not only of what is inoculated therein, but also of the characteristics of the selected hollow fiber.

            Thus, as used herein, a hollow fiber bioreactor or hollow fiber bioreactor cartridge consists of an outer

shell casing that is suitable for the growth of mammalian cells, a plurality of semi-permeable hollow fibers encased within the shell that are suitable for the growth of mammalian cells on or near them, and the EFS, which contains the cells and the EFS cell supernatant.

As used herein, tissue culture medium includes any culture medium that is suitable for the growth or maintenance of mammalian cells _in vitro_. Examples of such medium include, but are not limited to AIM-V, RPMI, and Iscove's medium (GIBCO, Grand Island, N.Y.).

As used herein, complete AIM-V is a tissue culture medium that consists of the proprietary formula AIM-V (GIBCO, Grand Island, N.Y.) and also contains 10 μg. gentamicin/ml. (GIBCO), 50 μg. streptomycin/ml. (GIBCO), 50 μg penicillin/ml. (GIBCO), 1.25 μg. fungizone/ml. (Flow Laboratories, MacLean, VA.).

Other suitable tissue culture media are well-known and readily available to those of skill in the art and may be readily substituted for AIM-V.

Hollow fiber bioreactors and hollow fiber culture systems.

Hollow fiber (hereinafter abbreviated as HF) bioreactors and HF cell culture systems are known to those of skill in the art (see, e.g., Knazek et al., U.S. Patent Nos. 4,220,725, 4,206,015, 4,200,689, 3,883,393, and 3,821,087, published international application WO 90/02171, which disclosures are herein incorporated by reference thereto). A HF cell culture system includes the HF bioreactor, pumping means for perfusing medium through the system, reservoir means for providing and collecting medium, and other components, including electronic controlling, recording and sensing devices.

25

A typical HF cell culture system, such as the CELLMAX™ 100 HF cell culture system (Cellco Advanced Bioreactors, Inc., Kensington, MD.), which is described in PCT International Application WO 90/02171, consists of
5   a standard glass media bottle, which serves as the reservoir, a stainless steel/Ryton gear pump, the autoclavable HF bioreactor, which includes the fibers and shell casing in which cells are cultured, and medical grade silicone rubber tubing, or other connecting means,
10   which serves as a gas exchanger to maintain the appropriate pH and pO_2 of the culture medium. All components are secured to a stainless steel tray of sufficiently small dimensions to enable four such systems to fit within a standard tissue culture incubator
15   chamber. The pump speed and automatic reversal of flow direction are determined by an electronic control unit which is placed outside of the incubator and is connected to the pump motor via a flat ribbon cable which passes through the gasket of the incubator door. The pump motor
20   is magnetically coupled to the pump and is lifted from the system prior to steam autoclaving. Tissue culture medium is drawn from the reservoir, pumped through the lumina of the hollow fibers, and then passed through the gas exchange tubing in which it is re-oxygenated and its
25   pH readjusted prior to returning to the reservoir for subsequent recirculation.

The HF bioreactor, which is a component of a HF cell culture system, is a cartridge that contains a multitude of semi-permeable tube-shaped fibers encased
30   within a hollow shell. The terms HF reactor and HF bioreactor are used interchangeably. HF bioreactors have been used for the growth of mammalian cells and for the production of biologically active products that are

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secreted thereby (see, _e.g._, Knazek _et al._ _supra._, see, also, Yoshida _et al._ U.S. Patent No. 4,391,912) and also have been used to cultivate viruses, including herpes simplex virus, hepatitis virus, equine encephalitis
5   virus, mouse mammary tumor virus and human immunodeficiency virus (see, _e.g._, Meyers _et al._ U.S. Patent No. 4,546,083; Markus _et al._, U.S. Patent No. 4,301,249; Johnson _et al._ (1978) _Appl. Envir._ 35:431; and Tsang _et al._ publication of Poster presentation at Bio-
10   Expo, 1986, Boston MA). HF bioreactors have, not however, heretofore been used for producing recombinant vectors, including recombinant viral vectors, in a form suitable for transducing target cells that are used in genetic therapy.

15   HF bioreactor cartridges, which are well known to those of skill in the art (see, _e.g._, PCT International Application WO 90/02171), contain a multitude of tube shaped semi-permeable membranes (hereinafter called fibers) that are encased in a hollow
20   shell. Cultured cells grow and fill the spaces between the fibers and are fed by passage of nutrients through the fiber walls from medium that is perfused through the lumina of the fibers. Any HF bioreactor known to those of skill in the art may be suitable for use to practice
25   this invention. Preferred HF bioreactors for use in accordance with this invention include the B3 and B4 bioreactors (Cellco Advanced Bioreactors, Inc., Kensington, MD) (see, _e.g._, PCT International Application WO 90/02171 for a complete description of the B3 and B4
30   bioreactors).

       The B3 bioreactor cartridge contains several thousand tube-shaped, semi-permeable membranes, which provide about a 1.1 m^2 surface area. The B4 bioreactor

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cartridge is somewhat larger than the B3 cartridge and
provides a fiber surface area of about 1.6 m². The
fibers, which are each approximately 250 μm in diameter,
are pulled through a polycarbonate tube that is about 12

5   inches in length, and the extra-fiber volume is filled at
each end with a polymeric material in a manner such that
liquid can flow through the lumina of the fibers to exit
at the opposite end of the shell. The fiber walls
nominally restrict passage to substances having molecular

10   weights less than a desired cut-off range. The selected
fiber should be semi-permeable to permit the passage of
nutrients into the EFS and should be of a material, such
as DEAE-cellulose or polypropylene, on which or in the
vicinity of which the mammalian cells are able to grow.

15   For example, the fibers used in the B3 and B4 cartridge
are cellulosic hollow fibers whose walls nominally
restrict diffusion to substances having molecular weights
in the range of 3000 to 4000 Daltons. This molecular
weight cut-off range is suitable for use in practicing

20   this invention because it is sufficiently small to
prevent diffusion of packaged recombinant viral vectors
out of the EFS. The fibers divide the cartridge into the
EFS, which is also referred to as the extra-capillary or
shell-side space, within which minimal bulk flow of

25   perfusion medium occurs by ultrafiltration through the
fiber wall. The EFS volume of the B3 cartridge is about
50 ml and that of the B4 cartridge is about 100 ml.

The particular cartridge selected for use
depends upon various parameters, including the

30   requirements of the cells that are being cultivated, the
materials perfusing through the lumina of the fibers, and
the cellular products and recombinant vectors that are
being harvested. It is within the level of skill in the

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art to select an appropriate bioreactor cartridge and also HF culture system. The fibers and, therefore, the cartridge and HF cell culture system, are selected as a function of the components of the perfusing medium to
5   which they must be permeable and/or impermeable and as a function of the components of the EFS. In accordance with this invention, the fibers will generally be selected such that they are impermeable to the viral vectors, packaged viral vectors or whatever form the
10   vectors are produced in order to maximize the concentration thereof in the EFS and to prevent undesirable contamination of the perfusing medium with such vectors or virions.

        Tissue culture medium perfuses through the
15   lumina of the fibers and is also included within the EFS surrounding said fibers. The tissue culture medium, which may differ in these two compartments, contains diffusible components that are capable of sustaining cell growth and proliferation. Tissue culture medium, which
20   is generally oxygenated, is provided in a reservoir from which it is pumped through the fibers. The flow rate can be controlled by the varying the pump speed. In addition, the direction of flow of the perfusing medium can be reversed (see, _e.g._, PCT International Application
25   WO 90/02171).

        The EFS and/or the perfusing medium may additionally contain an effective amount of at least one growth promoting or suppressing substance, such as IL-2, that specifically promotes the expansion or suppression
30   of the cultured cells, particularly the selected target cells, such as lymphocytes, in which the effective amount is an amount sufficient for the cells to be maintained or proliferate _in vitro_. The EFS and/or perfusing medium

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may also be supplemented with additional ingredients
including serum, serum proteins, and selective agents for
selecting genetically engineered or modified cells. The
selected method is a function of, among other variables,
5   the type of cells, their intended use, and the extent to
which they adhere to the fibers.

The flow rate can be increased as the number of
cells increases with time. Typically the initial flow
rate of the medium is adjusted to about 30 to 40 ml/min
10 and is then increased up to about 300 ml/min as the
number of cells increases with time. The direction of
perfusion of the medium through the lumina of the hollow
fibers may be periodically and automatically reversed,
typically every ten minutes, in order to provide a more
15 uniform distribution of nutrient supply, waste dilution,
and cells within the space surrounding the hollow fibers.

The entire system is sterilized prior to cell
inoculation and is designed for operation in a standard
air-CO_2 tissue culture incubator. Upon inoculation, the
20 cells settle onto the surface of the hollow fibers,
through which nutrients pass to feed the cells and
through which metabolic waste products pass to be diluted
into the large volume of the recirculating perfusate. A
suspension of cells is inoculated into the extra-fiber
25 space (EFS) of a HF bioreactor usually through one of two
side ports. The lumina are perfused with cell culture
medium and the cells are maintained _in vitro_ for the
desired period of time. As the cells are cultured, the
perfusing medium is periodically monitored for glucose
30 concentration. The perfusing medium is replenished by
replacing the medium in the reservoir bottles whenever
glucose concentration drops to about 30 to 40% of its
initial value.

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After culturing the cells, the EFS and/or the cells may be harvested. In accordance with this invention, the EFS from a bioreactor that contains producer cells is harvested and used to transduce target
5   cells that are cultured in a second bioreactor. After transduction, which may be repeated multiple times, and incubation, the target cells are harvested. Any suitable means know to those of skill in the art may be used to harvest the EFS and to harvest the target cells. For
10   example, in order to harvest the EFS, the bioreactor cartridge is removed from the incubator and placed in a laminar flow hood. The bioreactor cartridge containing the target cells is given a single gentle shake, which usually suspends about 30-40% of cells, and the contents
15   of the EFS, including the loosened cells, are drained into a side port bottle. Fresh medium is added to the EFS and incubation of the target cells can be continued.

Packaged vector production continues at high rate as long as the EFS is periodically harvested and
20   replaced with fresh medium. Other methods known to those of skill in the art for removing the EFS and cultured cells from the bioreactor may be used.

Selection of the recombinant viral vector and production
25   **of high titers thereof.**

The preparation and selection of the recombinant viral vector DNA encoding at least one gene product is within the level of skill in the art. In general, the selected recombinant viral vector is one that can be
30   replicated and packaged by selected producer cells but not by the selected target cells. It may be a vector that is integrated into a host cell genome, such as an SV40-derived or retrovirus-derived vector, or one, such

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as a vector derived from Epstein Barr virus, which includes an origin of replication, that remains episomal.

The gene product may be a therapeutic product, such as an anti-cancer or anti-viral agent; it may be a
5   product, such as adenosine deaminase or immunoglobulin, that the recipient either fails to produce or produces in a mutated defective form because of a genetic defect; it may be a marker, such as DNA that encodes neomycin or methotrexate resistance, whereby the reinfused target
10 cells may be selected or detected; or it may encode a product that regulates expression of another gene product.   Selection, cloning and insertion of the heterologous DNA into the recombinant viral vector is within the level of skill in the art and may be effected
15 by any of the well known methodologies therefor.

Any recombinant viral vector derived from viruses that can replicate in eukaryotic cells may be used. The selected heterologous DNA is inserted into the recombinant viral vectors, which is then introduced into
20 producer cells by any means known to those of the skill in the art.   The transfected producer cells are then cultured in a HF bioreactor for a time sufficient for replication and production of the recombinant vector, whereby high titers of recombinant vectors are produced.
25 Preferred embodiments employ recombinant retroviral vectors produced by a packaging cell line that secretes packaged replication-incompetent infectious retroviral particles into the EFS of the HF bioreactor.

Preferred retroviral vectors are those that are
30 suitable for genetic therapy. Suitability for use in genetic therapy necessitates minimizing the possibility for recombination to produce replication competent retrovirus or to activate cellular oncogenes and the

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retroviral vector must be packaged in packaging cell
lines that do not concomitantly produce helper virus.
Such retroviral vectors may be constructed by means known
to those of skill in the art may be retroviral vectors
5 known to those of skill in the art or may be derived
therefrom.

Typically, suitable retroviral vectors include:
the LTRs; necessary regulatory signals and retroviral
sequences to produce and integrate proviral DNA into the
10 host cell genome; and heterologous DNA, which includes
DNA that encodes a detectable marker and/or selectable
marker. The heterologous DNA is inserted in place of all
or portions of the retroviral structural genes in
operative linkage with transcriptional and translational
15 regulatory sequences including a promoter, such as the 5'
LTR or endogenous promoter, that is recognized by an RNA
polymerase in the target cell.

Retroviral vectors are generally constructed by
preparing cDNA, which is inserted into a convenient
20 plasmid, such as pBR322. Desired insertions and
deletions are effected using standard methods, and the
plasmids are introduced into selected packaging cell
lines in order to generate retroviral particles.
Introduction of the plasmids into the packaging cell line
25 may be effected by any method known to those of skill in
the art. For example, the DNA may be transfected by Ca-
phosphate mediated transfection (see, _e.g._, PCT
International Application WO 90/01870), DEAE-dextran
mediated transfection methods, lysozyme fusion, direct
30 uptake or any other method known to those of skill in the
art. Typically the plasmids are first introduced into an
ecotropic cell line to produce infectious packaged
particles, which are then transduced into an amphotropic

packaging cell line, and cultured in selective medium, and cellular clones are selected. The selected clones are tested for the ability to produce packaged retroviral vectors without concomitant helper virus production.

5 Those that do not produce helper virus and/or replication competent packaged retroviral vectors are suitable for use in producing packaged viruses for transducing target cells used in genetic therapy. Examples of retroviral vectors from which clinically useful recombinant

10 retroviral vectors that can be modified by insertion of heterologous include, but are not limited to, the retroviral constructs: pN2 (see, e.g., Keller et al. (1985 Nature 318:149-154; and U.S. Patent No. 4,861,719 to Miller); pLHL, which is derived from N2 (see, e.g.,

15 Miller et al. (1986) Cold Spring Harbor Symp. on Quantitative Biology, Volume LI, Cold Spring Harbor Laboratory , pp. 1013-1019); pSDHT (Miller et al. (1986) Somat. Cell. Mol. Genet. 12:175-183), which includes the bacterial marker gene that encodesthe neomycin phospho-

20 transferase gene (neo), which confers resistance to G-418; and pLPL (Proc. Nat'l Acad. Sci. USA 80: 4709-4713), which includes the gene encoding the selective marker hypoxanthine-guanine phosphoribosyltransferase, HGPRT.

25   Derivatives of these vectors, such as those that include the heterologous gene or genes of interest, may be constructed by inserting selected heterologous DNA into a retroviral vector in operative linkage with a promoter, which recognized by a target cell RNA

30 polymerase, and other transcriptional and translational regulatory signals. For example, retroviral vectors SSC and SSCX, ATCC Accession Nos. 67760 and 67761, respectively, which are derived from N2, encode a soluble

form of the glycoprotein receptor CD4, which as been
proposed for use in AIDS therapy. SSC and SSCX have been
packaged using the PA317 cell line (ATCC Accession No.
CRL 9078). The construct pLPL2 (see, _e.g._, U.S. Patent
5   No. 4,861,719 to Miller), which is derived from pLPL by
including additional deletions, such as deletion of the
second packaging signal in the 3' LTR, which prevents
packaging of pBR322 DNA.

        In a preferred embodiment, the retroviral vector
10 LASN (see Hock _et al._ (1989) _Blood_ 74: 876-881), which
is a derivative pLNL6, an N2 derivative (see Bender _et_
_al._ (1987) _J. Virol._ 61: 1639), and which encodes ADA,
is produced in high titer in a HF bioreactor by a
packaging cell line derived from PA317 (ATCC Accession
15 No. CRL 9078). The LASN plasmid vector includes DNA that
encodes 5' LTR, the psi$^+$ packaging signal, which includes
gag protein encoding sequences, but which have been
modified by changing ATG to TAG to prevent translation of
any _gag_ encoding sequences (see, _e.g._, Bender _et al._
20 (1987) _J. Virol._ 61: 1639); the cDNA encoding the ADA
gene inoperative linkage with the 5' LTR; the _neo_ gene
in operative linkage with the SV40 early region promoter
and enhancers; and the 3' LTR, which includes a
polyadenylation site. The AUG start codon for ADA mRNA
25 begins in the LTR, continues through ADA sequences, the
SV40 sequences and _neo_ sequences and terminates in the 3'
LTR. The LASN plasmid vector has been transfected into
the packaging line, PA317 (ATCC Accession No. CRL 9078),
which produces packaged LASN retroviral particles that
30 have an amphotropic host range (see, Hock _et al._ (1989)
Blood 74: 876-881) but which does not produce detectable
helper virus.

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In a preferred embodiment, the LASN-producing
PA317 cell line is inoculated into the EFS of a HF
bioreactor and cultured under conditions whereby packaged
LASN retroviral particles accumulate in the EFS medium at
5   high titer. No detectable helper virus is produced.

Preparation of producer cells.

Recombinant viral vectors used for genetic
therapy must be able to infect target cells, but should
10 not harm the host. Therefore, the viruses from which they
are derived should be modified such that they do not
commandeer target cell biochemical pathways to the
detriment of the target cell and/or host into which the
target cells are introduced. Consequently, in order to
15 amplify recombinant viral vectors that contain
heterologous DNA of interest, the recombinant vectors
must be cultured <u>in</u> <u>vitro</u> in producer cells in which they
can be replicated.

In preferred embodiments, recombinant viral
20 vectors are amplified in producer cells lines, called
packaging cell lines, that are cultured in a HF
bioreactor and secrete the recombinant vectors into the
EFS in a form in which the recombinant vectors can be
introduced into target cells. In particular, packaging
25 cell lines that produce infectious, replication-
incompetent, recombinant retroviral vectors that contain
heterologous DNA are cultured in a HF bioreactor and high
titers of packaged retroviral vector particles accumulate
in the EFS.

30   A cell line, such as PA317 that contains such
a DNA construct can transmit packaged viral RNAs,
including those that encode heterologous DNA, as long as
the viral RNA includes the proper cis-acting elements,

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such as the packaging signal. The packaged viral particles that are produced by PA317 are amphotropic, and, thus, can infect a broad range of mammalian target cells.

5   Suitable packaging cell lines may be constructed or may be derived from readily available well-known lines, which include, but are not limited to: psi^2 (Mann et al. (1983) Cell 33 153-159); NIH 3T3 TK⁻ (Miller et al. (1986) Mol. Cell. Biol. 6: 2895-2902); PA317 (ATCC
10 Accession No. CRL 9078) (see, e.g., U.S. Patent No. 4,861,719); and PE501, which is similar to PA317, but produces packaged retroviral particles that have an ecotropic host range (see, Hock et al. (1989) Blood 74: 876-881).

15   In a preferred embodiment, the retroviral vector LASN is produced by a packaging cell line that has been derived from PA317. Construction of PA317 is described in U.S. Patent No. 4,861,719 to Miller and construction of the LASN-producing derivative of PA317 is described in
20 Hock et al. (1989) Blood 74: 876-881.

          Briefly, DNA constructs, which were carried in pBR322 and in which all cis-acting elements, except for the tRNA binding side, had been deleted were introduced into NIH 3T3 TK⁻ cells by co-transfection with a
25 selectable marker, the HSV TK gene. TK+ cell clones were selected and tested forthe ability to package and transmit a retroviral vector, pLPL2, that includes the HGPRT gene in operative linkage with a promoter. The retroviral vector was introduced by calcium phosphate-
30 mediated transfection and the cells were cultured. After several days the overlying tissue culture medium was tested assayed for the presence of HGPRT-producing virus. The cell line, PA317, that packaged the highest titer of

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HGPRT-virus (6 x 10⁴ CFU/ml), was selected.

     The retroviral vector, LASN was constructed by introducing cDNA that encodes ADA was inserted in plasmid DNA that contained the retroviral LNL6, which is an N2-

5 derived vector (Bender _et al._ (1987) _J. Virol._ 61: 1639). In LASN, ADA-encoding mRNA begins in the 5'LTR, continues through ADA, SV40 and _neo_ sequences and terminates in the 3' LTR (see, _e.g._, Hock _et al._ (1989) _Blood_ 74: 876-881) The pBR322-derived plasmid that

10 contained the retroviral constructs was transfected into an ecotropic packaging cell line. Packaged retroviral particles from the ecotropic cell line were transduced into PA317 and G-418-resistant clones were selected and tested for helper virus production. Those that do not

15 produce helper virus have been used to produce packaged LASN for transduction of target cells.

     Other retroviral constructs containing heterologous DNA and packaging cell lines may be similarly constructed and used in accordance with this

20 invention.

Culturing producer cells in a HF bioreactor.

     Prior to use, a HF cell culture system, such as the CellMAX™ 100, is steam autoclaved, continuously

25 perfused with recirculating deionized water, drained, flushed, and perfused with the selected tissue culture medium in both the EFS and perfusate pathways. All operations are performed in sterile conditions, such as in a sterile laminar flow hood.

30   A sufficient amount, generally about 10⁵ - 2 x 10⁶ producer cells/ ml, of cells in a sufficient volume to fill the EFS of a bioreactor cartridge is inoculated into the pre-sterilized cartridge, such as a B3 or B4

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bioreactor cartridge (Cellco Advanced Bioreactors, Inc.,
Kensington, MD),. The inoculated bioreactor is
transferred to a standard incubator, perfused with
medium at an appropriate temperature, generally about 32°
5 C to about 37° C and maintained under these conditions.

After the cells settle, culture medium is
continuously perfused through the HF bioreactor by means
of externally applied pressure, such as a pump. A
reservoir that contains tissue culture medium, the HF
10 bioreactor cartridge, and pumping means are connected by
tubing, typically silicone rubber, which also serves as
an oxygenator. The medium may be oxygenated by any
means known to those of skill in the art. The silicone
rubber tubing simultaneously serves as a membrane gas
15 exchanger to replenish oxygen and, if the medium is
buffered with bicarbonate, to maintain the pH via CO_2
transport into the perfusion medium. Medium that is
buffered with systems other than bicarbonate do not
necessarily require CO_2 in the incubator.
20   Perfusion is continued for a sufficient time and
under conditions, whereby the vector is released into the
EFS, which then contains high titers of the recombinant
vector. The conditions, which include, the tissue
culture medium, incubation temperature and incubation
25 time, are chosen as a function of the requirements of the
producer cells and recombinant viral vector.
Determination and optimization of such conditions are
within the level of skill in the art.

During the incubation period, the reservoir
30 containing the perfusing medium is replaced in order to
maintain a sufficiently high concentration of glucose and
other diffusible nutrients in the EFS and for waste
removal. Typically, the perfusate is replenished several

times a week by replacing the reservoir bottle with one
containing fresh medium. Incubation continues for at
least about one to thirty days or more days. During the
incubation period, the EFS is periodically harvested and
5 contacted with target cells. After the cells have been
incubating for one to several days to a week or so, the
EFS can be harvested.   The EFS can be harvested
batchwise, periodically, or continuously or by any
variation thereof known to those of skill in the art.
10 It may also be connected to the EFS of a second
bioreactor that contains target cells, as shown, for
example, in Fig. 2.

In preferred embodiments, LASN-producing PA317
cells, which are suspended in tissue culture medium, are
15 inoculated into a B3 or B4 bioreactor, (Cellco Advanced
Bioreactors, Kensington, MD) via the side ports. The
bioreactor is attached to a perfusion circuit, the cells
are permitted to settle onto the fibers for about 15
minutes to several hours before perfusion is initiated.
20 After perfusing overnight or for day or two the EFS can
be harvested, batchwise or by directly introducing it
into a second bioreactor using a dual perfusion circuit,
fresh EFS medium is added and perfusion continues. If
the EFS is harvested periodically, about once a day, the
25 LASN-producing cells continue to produce high titers, at
least about 10^5 CFU/ml, for up to at least 6 to 7 weeks.

Viral titer may be measured by any method known
to those of skill in the art (see, e.g., U.S. Patent No.
4,861,719 to Miller; and U.S. Patent No. 4,868,116 to
30 Morgan et al. Typically viral titer is measured as
colony forming units/ml.

Selecting target cells and transducing them with recombinant viral vectors produced by cells cultured in a HF bioreactor.

Target cells, such as fibroblasts (see, e.g.,
5   Palmer et al. (1987) Proc. Natl. Acad. Sci. 84: 1055; St.
Louis et al. (1988) Proc. Natl. Acad. Sci. 85: 3150-54
and PCT International Application WO 90/01870) epithelial
cells (see, U.S. Patent No. 4,868,116 to Morgan et al.)
and immune cells, such as lymphocytes, are obtained
10   either from the patient, who has the inherited or
acquired disease or from another donor. The selected
target cells are contacted with the harvested recombinant
viral vectors that were produced in a HF bioreactor to
produce transduced target cells. In order to enhance
15   infectivity of the viral vector, polycations, such as
protamine at concentrations of about 5-10 μg/ml, may be
added to the harvested viral vectors or to the bioreactor
in which the producer cells are cultured. Contacting may
be effected by any method known to those of skill in the
20   art. The target cells are then inoculated into the EFS
of a second bioreactor, which has been autoclaved and
prepared as described above for the producer cells, and
incubated as described above.

The target cells may be transduced, either
25   before or after inoculation into the EFS of the second
bioreactor, by contacting with the EFS medium from a
first bioreactor that contains producer cells. The
target cells may be mixed with harvested EFS medium, may
be introduced into a second bioreactor into which the
30   harvested EFS medium is inoculated or the EFS of the
second bioreactor may be connected to the EFS of a first
bioreactor that contains producer cells and is
continuously or intermittently inoculated. The target

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cells are contacted with harvested vector-containing EFS medium one or more times.

Target cells can be harvested by gently shaking the bioreactor and pouring the suspended cells into a
5   side port bottle. Generally about 10^{10} target cells are used for one treatment and, ideally, 100% should be transduced.

In preferred embodiments, lymphocytes are inoculated into a second bioreactor and then transduced
10   with the EFS medium from a first bioreactor, which contains packaged infectious replication-incompetent retroviral vectors.

In a preferred embodiment, transduction is effected continuously or intermittently using a dual
15   perfusion circuit as shown in Fig. 2, discussed below, by connecting the EFS of the second bioreactor to EFS of a first bioreactor in which retroviral vector-producing cells have been inoculated. The target cells are then repeatedly exposed to EFS medium from the first
20   bioreactor. A high percentage of target cells can thereby be transduced.

The dual bioreactor perfusion circuit.

Continuous or intermittent inoculation of target
25   cells with recombinant retroviral vectors may be effected by directly pumping the EFS from the first bioreactor that contains the producer cells into the EFS of the second bioreactor that has been inoculated with target cells. This may be accomplished using the dual perfusion
30   circuit, pictured in Fig. 2.

As shown in Fig. 2, the perfusion circuits of the two bioreactors are separated by a pinch clamp (represented by ⋈ in Fig. 2). A peristaltic pump, is

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used to introduce fresh medium into the bioreactor that
contains the producer cells. When the peristaltic pump
is operating, the pinch clamp opens, thereby connecting
the EFS of the first bioreactor via a side port to that
5   of the second bioreactor via a side port. EFS medium
that contains recombinant retroviral vectors is forced
out of the first bioreactor through the connecting tubing
and pinch clamp and into the second bioreactor. Fresh
medium is introduced through the second side port of the
10 producer bioreactor. A filter that is designed to remove
blood leukocytes, is placed in the tubing between the two
bioreactors, interposed between the pinch clamp and the
second bioreactor. This prevents contamination of the
target cells by producer cells. Excess medium from the
15 EFS of the second bioreactor is forced through the second
side port into an overflow flask. The peristaltic pump
may be operated continuously or periodically. In
preferred embodiments, the pump is operated for about 1
minute per hour at a pressure sufficient to introduce
20 about 10 ml of vector-containing EFS medium into the EFS
of the second bioreactor that contains target cells.

         The following examples are included for
illustrative purposes only and are not intended to limit
the scope of the invention.

25   **EXAMPLE 1**
Production of ADA-containing retroviral particles.

         LASN-producing cells were the gift of Dr. Dusty
Miller. The construction of the LASN retroviral vector
that contains the ADA gene in operative linkage with the
30 LTR and the helper-free cell line that produces packaged
LASN retroviral vector particles is described in U.S.
Patent No. 4,861,710 to Miller and in Hock et al. (1989)
BLood 74: 876-881 is discussed above. The packaging cell

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line and LASN vector were prepared using publicly available and well known starting materials.

Briefly, LASN is a derivative of the vector LNL6 (Bender et al. (1987) J. Virol. 61: 1639), which is a
5   derivative of the well-known vector N2 (see, Nature 318: 149-154; see, also, Armentano et al. (1987) J. Virol. 61: 1639). LASN includes, starting at the 5' LTR, which is the MoMLV LTR, the extended packaging signal, psi$^+$, ADA cDNA under control of the LTR, the SV40 early region
10   promoter and enhancer, the bacterial neo gene under control of the SV40 promoter, the second LTR, and a polyadenylation site. ADA encoding sequences extend through the SV40 and neo and into the 3' LTR. A plasmid containing the LASN sequences was introduced into an
15   ecotropic packaging cell line, PE501, and virus from these cells was used to infect PA317, ATCC accession no. CRL 9078, and G-418-resistant LASN-producing PA317 cells were isolated.

LASN-producing PA317 cells were grown in T-150
20   flasks and cultured to produce confluent monolayers. The cells from three T-150 culture flasks (about 4.5×10^7 LASN-producing PA317 cells) were trypsinized, resuspended in complete tissue culture medium (cTCM), which contains 10% heat-inactivated fetal calf serum (FCS) (Hyclone,
25   Logan, UTAH), and centrifuged at 800 x g for about 10 minutes at room temperature. The cell pellet was resuspended in 100 ml cTCM and inoculated into the EFS of a B4 HF cartridge (Cellco Advanced Bioreactors, Kensington, MD) via the side ports. All operations were
30   performed in a sterile laminar flow hood.

Prior to use, the silicone rubber tubing flow path from the bioreactor culture system had been conn cted to the pump and reservoir and steam autoclaved

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with side port tubing and bottles at about 121° C for 20
minutes.   Using sterile technique, a B4 bioreactor
cartridge was removed from its package and inserted into
the sterilized silicone rubber tubing pathway. The side
5   port bottles were attached to the side ports of the B4 HF
cell bioreactor. The distilled water in the EFS of the
bioreactor was drained into empty side port bottles and
discarded.   The system was perfused with 0.8 liters of
deionized water overnight at 37° C. The perfusion pathway
10   and extra-fiber space of each system were then drained
and flushed with Dulbecco's minimal essential tissue
culture medium (DMEM, Gibco, Grand Island, NY) which was
then discarded and replaced with cTCM, which contained 45
gm glucose/liter DMEM, 10% heat-inactivated FCS, 50 units
15   of penicillin/ml, 50 μg streptomycin/ml, and 2.5 μg
amphotericin B/ml, which had been placed in the reservoir
of the perfusion circuit. The bioreactor culture system
was then transferred to a standard tissue culture
incubator, which was held at 37° C and contained a
20   humidified 5% CO_2 in air atmosphere.   Perfusion was
initiated at a rate of about 100 ml/minute. After an
overnight perfusion, the bioreactor was removed from the
incubator and the EFS was inoculated with 100 ml of the
resuspended LASN-producing PA317 cells via the side port
25   bottles.   The entire CELLMAX™ bioreactor unit was then
placed into the incubator, but not perfused for 4 hours,
in order to facilitate uniform attachment of cells to the
fibers. Subsequent perfusion was commenced at a rate of
about 40 ml per minute and gradually increased to about
30   300 ml per minute during the course of the culture in
order to insure that the cells were adequately
oxygenated.   The direction of flow of perfusing medium
was not reversed.

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Glucose concentration of the perfusing medium was monitored about every 1-4 days. The perfusion medium was replaced when glucose concentration had dropped to about 30-50% of the initial value of about 4.5 gr/l. The
5   medium replacements were performed in a laminar flow hood.

As expected, the perfusate, which was assayed for virus by a colony forming assay, contained no detectable virus. The viral particles are about 150-200
10   nm in diame-ter, which is too large to diffuse through the fiber walls. At the indicated times (see Figures 1a and 1b), the medium in the EFS, which contained the viral particles, was harvested and replaced with fresh cTCM. The harvested medium was stored at -70 to -80° C awaiting
15   assay for virus content.

Figure 1a presents viral titer as a function of days in culture in the bioreactor. Figure 1b presents the total number of viral particles produced per day as a function of days in culture.

20   **EXAMPLE 2**
The effect of harvesting and replacing the EFS on total viral output and viral titer/ml.

On two successive days, day=t and day=t+1, the EFS medium from a bioreactor, which had been inoculated
25   and incubated as described in Example 1, was harvested and titered. The EFS was harvested by gently pressurizing one side port bottle through a 0.2 micron filter to force the contents of the EFS out of the bioreactor and into the other side port bottle.

30   The first EFS harvest, occurring 22.5 hours after the EFS medium was introduced, yielded a titer of 7.0×10^5 CFU/ml. This was equivalent to a production rate of 5.2×10^7 virus particles per day. After the

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harvest in which all of the EFS medium was removed, fresh medium was added to the EFS and incubation was continued for another 4 hours, after which the all of the EFS medium was harvested and titered. This titer was 2×10^5,

5   which is equivalent to production rate of 12.0×10^7 particles per day. All remaining EFS medium was removed and completely replaced by fresh medium. The EFS medium was then harvested after 17 hours and completely replaced with fresh medium, which was harvested after 7 hours.

10 The results are shown in TABLE I.

It was possible to achieve viral titers of 10^5-10^6 and viral production rates from a single bioreactor of more than 10^8 viral particles per day. Furthermore, increasing the frequency of the EFS harvest appeared to

15 increase total viral output.

For comparison, two T-150 flasks containing a pre-confluent monolayer (about 10^7 cells) of LASN-producing PA317 cells were incubated at 37° C in a humidified 5% CO_2 atmosphere. When the cells were

20 confluent, the medium from the monolayer cultures was harvested and titered. The results, which are presented in TABLE II, demonstrated that in monolayer culture, it was only possible to achieve a titer in the range of about 10^3 particles/ml.

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TABLE I

PRODUCTION OF VIRAL PARTICLES IN THE HF BIOREACTOR

Conse-cutive Samples	Duration hours*	titer $\times 10^{-5}$**	EFS volume	rate of virus production $\times 10^{-7}$***
1	22.5	7.0	70	5.2
2	4.0	2.0	100	12.0
3	17.0	5.0	75	5.3
4	7.0	8.9	70	21.3

*	hours between replacing and harvesting the EFS
**	titer= recombinant viral particles/ml
***	rate of recombinant viral particle production = recombinant viral particles/ml/day x EFS medium volume.

TABLE II

PRODUCTION OF VIRAL PARTICLES IN MONOLAYER CULTURE

Flask#/ harvest#	Duration hours*	titer $\times 10^{-3}$**	volume ml	rate of virus production $\times 10^{-5}$***
1/1	29	1.67	92	1.27
1/2	27	1	90	.80
2/1	29	1.11	92	.84
2/2	27	.56	90	.44

* medium	hours between feeding and harvesting all of the in the flask
**	titer= recombinant viral particles/ml
***	rate of recombinant viral particle production = recombinant viral particles/ml/day x volume of supernatant.

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EXAMPLE 3
Transduction of lymphocytes with the retroviral particles.

A LASN-producing PA317 culture was initiated by
5 inoculating a B3 bioreactor (Cellco Advanced Bioreactors,
Inc. Kensington, MD) with 8.4×10^8 cells, which were 100%
viable. The B3 cartridge is similar to the B4 cartridge,
except that it smaller. The EFS has a volume of about
50 ml. Tri-lumen, thin-walled tubing was used to connect
10 the cartridge outlet to reservoir in order to increase
oxygen transport into the medium. The direction of
perfusion of medium was periodically reversed as
described in PCT International Application No. WO
90/01271.

15   The B3 bioreactor was perfused with DMEM that
contained 10% heat-inactivated FCS, 2 mM glutamine (Flow
Laboratories), 50 units of penicillin/ml and 50 μg
streptomycin/ml. The unit was placed in the incubator,
as described in Example 1, and the cells were permitted
20 to attach for about 15 minutes. Perfusion was commenced
at a low flow rate, less than 100 ml/min. and gradually
increased to 300 ml/min.   The EFS was harvested
periodically as described in Example 2 and centrifuged at
about 800 x g for about 10 min. to remove cells and cell
25 debris. The harvested EFS was stored at -70 – $-80°$ C for
later analysis.

About five weeks after the culture was
initiated, about 60 ml. was harvested from the EFS and
replaced with fresh medium.   About 10 ml. of the
30 harvested EFS was removed, diluted and checked for
bacterial contamination, which was absent, and to check
the cells, which appeared healthy.

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The remaining 50 ml. was filtered through a 1μ pore size nylon Polydisc™ AS filter (WHATMAN Ltd., Maidstone, England). This filtrate was used for transduction of the target cells.

5   The selected target cells were a human T cell lymphotropic virus I (HTLV I)-transformed, ADA-deficient, IL-2-dependent, human lymphocyte cell line, TJF-2. TJF-2 was originally obtained from a patient having ADA deficiency and was then transformed with HTLV I.

10   A second bioreactor, a B4 bioreactor (Cellco Advanced Bioreactors, Inc., Kensington, MD) was inoculated with 1. 3 x 10^8 TJF-2 cells. The cells were cultured in the bioreactor in RPMI 1640 tissue culture medium (Biofluids, Rockville, MD.) supplemented with 10%
15 heat inactivated FCS, penicillin, streptomycin, 2 mM glutamine (Flow Laboratories) and 1000 units/ml of interleukin-2 (IL-2) (provided by Cetus Corp., Emeryville, CA). 1000 units/ml of IL-2 were also included in the EFS. The medium in the reservoir was
20 changed every 1-4 days. Also, more TJF-2 cells were periodically injected into the EFS in order to increase cell density. After the culture was established, 7.2 x 10^8 cells were removed in a laminar flow hood, by giving the bioreactor cartridge a single gentle shake. About
25 30-40% of cells were then drained into a side port bottle. The cells were about 65% viable. About 20% of the cells were removed to serve as non-transduced controls. The remaining cells were pelleted and resuspended in 45 ml of the LASN filtrate, described
30 above.

The EFS of the second bioreactor, which contained the remaining TJF-2 cells, was reinoculated with the resuspended cells. RPMI 1640 complete m dium,

prepared as described above, was added to fill the EFS, the unit was placed in the incubator and perfusion was started. After 2 days, the transduction procedure was repeated with a second volume of LASN filtrate. After

5 two more days, the transduction procedure was repeated again. Before each transduction with LASN, samples of the cells were removed for analysis. The culture was terminated after about 8 days. It contained a total of 1.02×10^{10} cells with 82% viability.

10   Some of the sample transduced cells were introduced into soft agar and cultured in the presence of G-418. Colony formation indicated that the cells were expressing the neo gene that is present in the LASN vector.

15   In addition, samples of TJF-2 cells taken from the second bioreactor before transduction, two days after the first transduction and two days after the second transduction were assayed for the presence of the neo gene by polymerase chain reaction (PCR) analysis, which

20 demonstrated the presence of the gene after transduction.

       PCR analysis was carried using the GeneAmp[R] Reagents and DNA Thermal Cycler (Perkin Elmer Cetus, Emeryville, CA). DNA was isolated from the transduced and non-transduced TJF-2 cells and PCR was initiated with

25 1-2 μg of genomic DNA with neo gene primers having the following sequences:

                CAAGATGGATTGCACGCAGG

                CCCGCTCAGAAGAACTCGTC.

       The reaction mixture was heated at 94° C for 2

30 min., annealed at 56° C for 2 min, and extended at 72 ° C for 3 minutes in the DNA Thermal Cycler for 30 repetitive cycles. The products of the reaction were run on a gel and probed with a neo-specific probe. Genomic

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DNA from the transduced cells included <u>neo</u> DNA; whereas the non-transduced cells did not. Therefore, the target cells are transduced by LASN and have LASN-derived DNA incorporated into genomic DNA.

5   **EXAMPLE 4**

Transduction of primary, non-transformed, ADA-deficient lymphocytes with LASN using a dual perfusion bioreactor circuit.

A   B3   cartridge,   prepared   as   described   in
10 Example 1, was inoculated with 3.5 x 10^8 primary, non-transformed, ADA-deficient lymphocytes, and suspended in 84 ml of the continuously harvested LASN-containing EFS medium. The LASN-containing EFS medium had been harvested during the previous 48 hrs and supplemented with 1000
15 units of IL-2/ml (Cetus, Emeryville, CA). The bioreactor was perfused overnight with AIM-V containing 1000 U IL-2/ml in the forward direction at a rate of about 100 ml/min.

The   EFS   of   the   first   bioreactor,   which
20 contained   the   LASN-producing   PA317   cells,   was   then connected to the EFS of the target cell bioreactor in the arrangement of a dual perfusion circuit as shown in Fig. 2.   A peristaltic pump pumps fresh medium, DMEM medium containing   10% FCS (HyClone, Logan, UTAH) and 1000
25 units/ml IL-2, into the entrance side port in the first bioreactor. The perista pump was run for 1 minute every hour. The fresh medium was pumped into the EFS at a rate of 5 mls/min.   The LASN-producer PA317 cartridge is perfused with DMEM that contains 2% FCS at a rate of 300
30 ml/min. As result of the relatively high flow rate of the perfusate and intermittently open side ports, some of the perfusate was ultrafiltered into the EFS through the fiber walls at an approximate rate of about 5 ml/min.

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Since serum proteins do not ultrafilter to a significant extent, the final concentration of FCS proteins in the EFS is estimated to be about 5% FCS. When fresh EFS medium is not being pumped into the side ports, the perfusate is not ultrafiltered.

The pinch clamp that separates the EFS of the two bioreactors automatically closes when the pump is off. A RC-50 Pall filter (Pall Biomedical Products Corp., Glen Cove, NY), which is a leukocyte removal filter for blood, is interposed between the first bioreactor and the clamp in order to remove any LASN-producing cells that might be dislodged from the EFS of the bioreactor that contains the LASN-containing EFS medium.

The two bioreactors were sterilely attached using a Sterile Tubing Welder (SCDIIB, DuPont, Wilmington, DE). One side port of the second bioreactor is connected to the input from the EFS of the first bioreactor and the other is connected to a flask, which collects the overflow.

About 3.5 x 10^8 lymphocytes were inoculated into the second bioreactor and perfused overnight with AIM-V that contained 1000 units/ml of IL-2 at a rate of about 50 ml/min that reversed direction every minute. Because the flow rate of the perfusate in the second bioreactor was relatively slow, there was no substantial ultrafiltration into the overflow flask.

About 19 hours after the second bioreactor was inoculated, the peristaltic pump, which was pumping at a rate of about 5 ml/min, was turned on for one minute and about 10 ml of the EFS medium from the first bioreactor was introduced into the EFS of the second bioreactor. The 10 ml includes 5 ml of fresh medium from the

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reservoir and 5 ml resulting from the ultrafiltration of
the perfusate into the EFS that occurs by virtue of the
relatively high flow rate of the perfusate. Incubation
was continued and once an hour the peristaltic pump was
5   automatically turned on for 1 minute. After about 87
hours in culture, including the initial 19 hours,
approximately 0.5×10^8 lymphocytes from the second
bioreactor were harvested by draining them from the EFS.

At the same time, the second bioreactor was
10 inoculated with the lymphocytes, 2×10^6 lymphocytes were
introduced into each well of two 24 well Costar™ plates.
Prior to introduction into the plates, the lymphocytes
had been suspended in 1 ml of the continuously harvested
LASN-containing EFS medium, which was harvested as
15 described in Examples 1 and 2. Then 1 ml of AIM-V
containing 2000 units/ ml of IL-2 was added to each well.
The cells were incubated in a CO_2 incubator at 37° C for
87 hours. About 1.6×10^8 cells were harvested from the
plates at the same time the lymphocytes in the second
20 bioreactor were harvested.

Aliquots of about 10^7 lymphocytes from both the
plates and bioreactor were placed in centrifuge tubes,
pelleted at 800 x g for 10 min, washed in Hanks BSS, re-
pelleted and frozen at -80° C for subsequent ADA protein
25 analyses.

EXAMPLE 5

**Measurement of ADA production by the transduced
lymphocytes.**

Samples of lymphocytes prepared in Example 4
30 were assayed for ADA production. The cell pellets were
warmed and lysed by freeze-thawing. ^{14}C-adenosine, an ADA
substrate, was added and the mixture was incubated at 37°
C for 1 hr, heat at 95° C for 5 min to quench the

reaction. The mixture is centrifuged and aliquots are spotted onto thin layer chromatography (TLC) paper and run in solvent containing Na-phosphate, saturated ammonium sulfate and N-propyl alcohol for an 1 hr. The

5 TLC paper dried overnight. The spots are cut-out and placed scintillation vials with scintillation fluid and counted in a scintillation counter.

The rate of ADA production by the singly transduced lymphocytes grown in the 24 well plates was

10 55.2 nmoles/min/10^8 lymphocytes. ADA production by the continuously transduced lymphocyte cells harvested from the second bioreactor was 73.2 nmoles/min/10^8 lymphocytes. This rate of ADA production is comparable to the rate of ADA production, 50-90 nmoles/min/10^8 lymphocytes, by

15 normal lymphocytes that are not deficient in ADA production.

In comparison, the rate of ADA production by lymphocytes transduced using the supernatant obtained from LASN-producing PA317 cells grown in monolayer and

20 exposed to the supernatant six times was only 11.6 nmoles/min/10^8 lymphocytes. The ADA activity of the non-transduced lymphocytes from the ADA-deficient patient was 0.6 nmoles/min/10^8 lymphocytes.

Since modifications will be apparent to those of

25 skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

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We claim:

1. A method for producing a high titer of recombinant viral vectors, comprising:

(a) inoculating the extra fiber space
5 (EFS) of a hollow fiber bioreactor with producer cells that release recombinant viral vectors into the EFS medium; and

(b) incubating said producer cells in said bioreactor under conditions whereby the titer of said
10 recombinant viral vectors is sufficiently high to transduce target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell.

2. The method of claim 1, further comprising harvesting said recombinant viral vectors by collecting
15 the EFS medium.

3. The method of claim 2, further comprising introducing fresh medium into the EFS of said bioreactor and incubating said producer cells under conditions whereby the titer of said viral vectors in said fresh
20 medium is sufficient to infect target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell.

4. A method for producing a high titer of packaged recombinant viral vectors, comprising:
25 (a) inoculating the extra fiber space (EFS) of a hollow fiber bioreactor with packaging cell line that releases packaged recombinant viral vectors into the EFS; and

(b) incubating said cells in said
30 bioreactor under conditions whereby the titer of said packaged recombinant viral vectors is sufficient to infect target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell.

5.   The method of claim 4, further comprising
harvesting said vectors by collecting said EFS medium.

6. The method of claim 4, further comprising
introducing fresh medium into the EFS of said bioreactor
5   and incubating said packaging cells under conditions
whereby the titer of said viral vectors in said fresh EFS
is sufficient to infect target cells at a multiplicity of
infection of more than 1 recombinant viral vector per
target cell.

10   7.   The method of claim 6, further comprising
repeating said harvesting, introducing, and incubating
steps a plurality of times.

8.   A method for producing a high titer of
recombinant viral vectors, comprising:

15   (a)   inoculating the extra fiber space
(EFS) of a hollow fiber bioreactor with a producer cell
line that releases recombinant viral vectors into the EFS
medium; and

(b)   incubating said producer cells in said
20 bioreactor under conditions whereby the titer of said
vectors in the EFS medium is at least about 10-fold
higher than that produced by said cells when they are
cultured in monolayer culture.

9.   The method of claim 8, wherein said titer is
25 at least about 10^5 colony forming units/ ml.

10.   The method of claim 8, further comprising
harvesting said vectors by collecting said EFS medium.

11. The method of claim 10, further comprising
introducing fresh medium into the EFS of said bioreactor
30 and incubating said cells under conditions whereby the
titer of the recombinant viral vectors in the EFS medium
is at least about 10-fold higher than that produced by
said cells when they are cultured in monolayer culture.

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12.   The method of claim 11, further comprising repeating said harvesting, introducing, and incubating steps a plurality of times.

13.   A method for producing a high titer of
5   recombinant retroviral vectors, comprising:

(a)   inoculating the extra fiber space (EFS) of a hollow fiber bioreactor with a packaging cell line that releases recombinant retroviral vectors into the EFS medium; and

10   (b)   incubating said producer cells in said bioreactor whereby the titer of said vectors is at least about 10-fold higher than that produced by said cells when they are cultured in monolayer culture.

14.   The method of claim 13, wherein said titer
15   is at least about 10^5 colony forming units/ ml.

15. The method of claim 7, wherein the rate of production of said packaged vectors is greater than about 10^5 CFU/ml/day.

16.   The method of claim 4, wherein said vectors
20   are recombinant retroviral vectors.

17.   The method of claim 16, wherein said retroviral vector is LASN.

18.   The method of claim 13, wherein said vector is LASN.

25   19.   The method of claim 4, wherein said packaging cell line is derived from PA317.

20.   A method of producing a high concentration of transduced target cells, comprising

(a)   inoculating the extra fiber space
30   (EFS) of a first hollow fiber bioreactor with producer cells that release recombinant viral vectors into the EFS medium;

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(b) incubating said producer cells in said bioreactor for a time sufficient to produce a titer of said recombinant viral vectors in said EFS medium that is sufficiently high to infect target cells at a

5 multiplicity of infection of more than 1 recombinant viral vector per target cell; and

(c) contacting target cells with said EFS medium under conditions whereby said vectors transduce said target cells.

10   21. The method of claim 20, wherein said vectors are recombinant retroviral vectors.

        22. The method of claim 21, wherein said recombinant retroviral vector is LASN.

        23. The method of claim 20, wherein said

15 contacting is effected by mixing the EFS medium that contains the recombinant vectors with said target cells.

        24. The method of claim 23, wherein said contacting is repeated plurality of times.

        25. The method of claim 24, further comprising

20 introducing said target cells into a second hollow fiber bioreactor and incubating said cells under conditions whereby a therapeutically effective amount of transduced target cells are produced.

        26. The method of claim 20, wherein said

25 contacting is effected by inoculating the EFS of a second bioreactor that contains target cells with the EFS medium from the first bioreactor that contains the producer cells.

        27. The method of claim 26, further comprising

30 incubating said target cells under conditions whereby a therapeutically effective amount of transduced target cells are produced.

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28. The method of claim 26, further comprising incubating said target cells under conditions whereby said cells are at least maintained; removing the EFS medium from said second bioreactor; and repeating said inoculating step.

29. The method of claim 26, wherein said removing and inoculating steps are repeated a plurality of times until a therapeutically effective amount of target cells are produced.

30. The method of claim 26, wherein said removing and inoculating steps are repeated a plurality of times until substantially all of said target cells are transduced.

31. The method of claim 26, wherein said EFS of said second bioreactor is inoculated with said EFS medium from the first bioreactor by connecting said bioreactors in a dual perfusion circuit, whereby the EFS of the first bioreactor is connected to the EFS of the second bioreactor.

32. The method of claim 31, wherein said inoculation is effected intermittently.

33. The method of claim 31, wherein said inoculation is effected continuously.

34. The method of claim 20, wherein said producer cells are a retroviral vector packaging cell line.

35. The method of claim 1, wherein said recombinant viral vectors include heterologous DNA that encodes a therapeutically effective product.

36. The method of claim 4, wherein said recombinant viral vectors include heterologous DNA that encodes a therapeutically effective product.

37. The method of claim 20, wherein said recombinant viral vectors include heterologous DNA that encodes a therapeutically effective product.

38. The method of claim 36, wherein said product is selected from the group consisting of adenosine deaminase, tumor necrosis factor, factor VIII, factor IX, interleukin-2, soluble CD4 glycoprotein receptor protein, an antibody, glucose cerebrosidase and the normal product of the gene responsible for cystic fibrosis, Tay Sachs, or Duchenne muscular dystrophy.

39. The method of claim 37, wherein said product is selected from the group consisting of adenosine deaminase, tumor necrosis factor, factor VIII, factor IX, interleukin-2, soluble CD4 glycoprotein receptor protein, an antibody, glucose cerebrosidase and the normal product of the gene responsible for cystic fibrosis, Tay Sachs, or Duchenne muscular dystrophy.

40. The method of claim 20, wherein said packaging cells are derived from PA317.

41. The method of claim 20, wherein said target cells are selected from the group consisting of fibroblasts, immune cells and epithelial cells.

42. The method of claim 41, wherein said cells are lymphocytes.

43. A composition, comprising transduced target cells, wherein said target cells are transduced with recombinant retroviral vectors and the concentration of said transduced target cells is greater than 10%.

44. The composition of claim 43, wherein said target cells are selected from the group consisting of fibroblasts, immune cells, and epithelial cells.

45. The high concentration of transduced target cells produced by the method of claim 20, wherein the

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percentage of transduced target cells is greater than 10%.

46. The high concentration of transduced target cells produced by the method of claim 31, wherein the percentage of transduced target cells is greater than 10%.

47. The high concentration of transduced target cells produced by the method of claim 30.

48. A composition, comprising a therapeutically effective concentration of transduced target cells.

49. A dual bioreactor perfusion circuit, comprising:

a first hollow fiber bioreactor and a second hollow fiber bioreactor, wherein the extra fiber space (EFS) of said first bioreactor is connected to the EFS of said second bioreactor by means for intermittently opening said circuit and introducing the EFS medium of said first bioreactor into the EFS of said second bioreactor.

50. The dual bioreactor perfusion circuit of claim 49, further comprising filtering means interposed between said bioreactors for preventing introduction of any cells from the EFS said first bioreactor into the EFS of said second bioreactor.

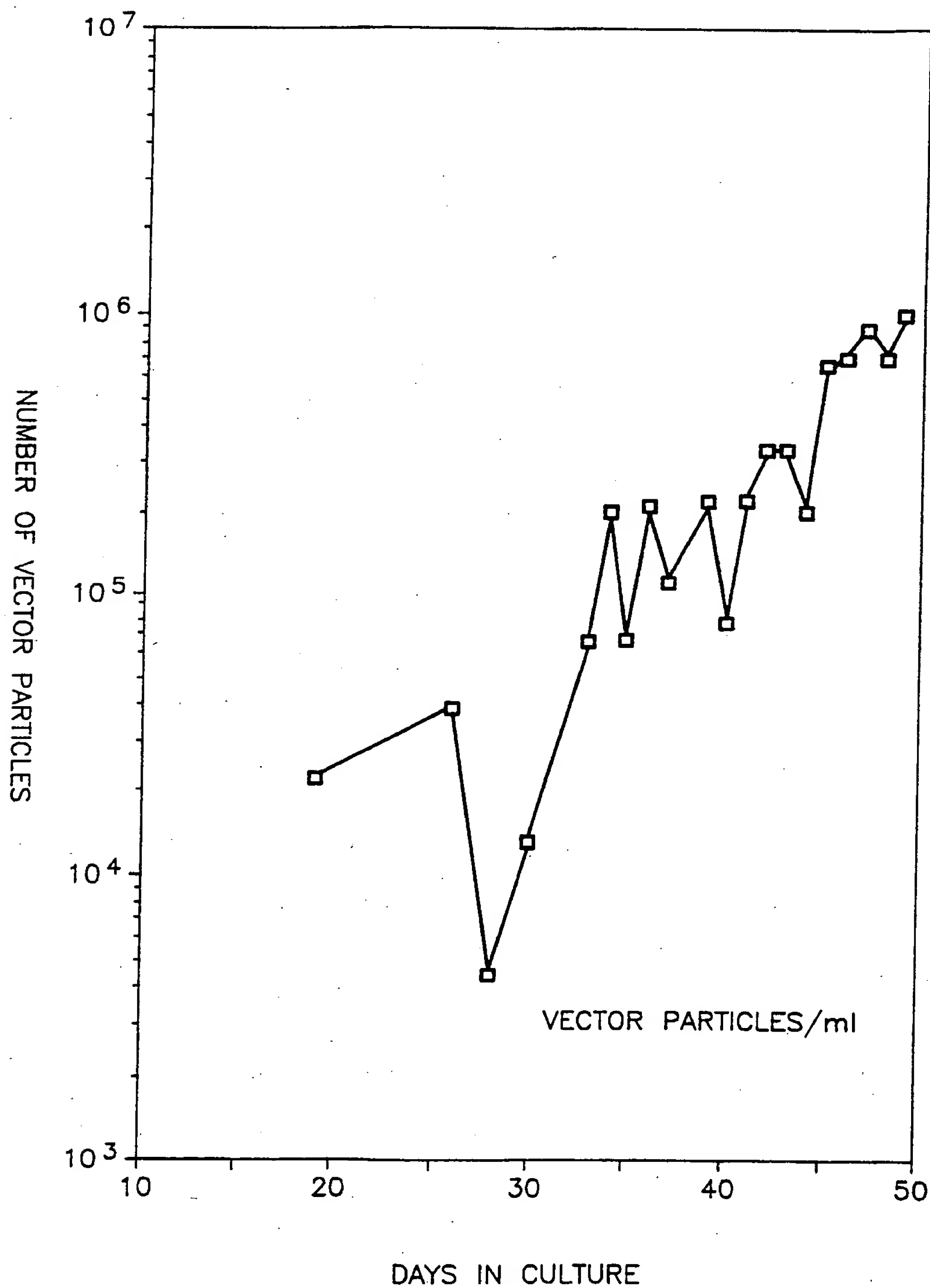51. A method of producing a high concentration of transduced cells, comprising:

culturing said cells in a hollow fiber bioreactor and continuously introducing a suspension of recombinant viral vectors into the EFS of said bioreactor, whereby a substantial percentage of said cells are transduced by said vectors.

52.   The method of claim 51, wherein said recombinant viral vectors are derived from viruses that infect eukaryotic cells.
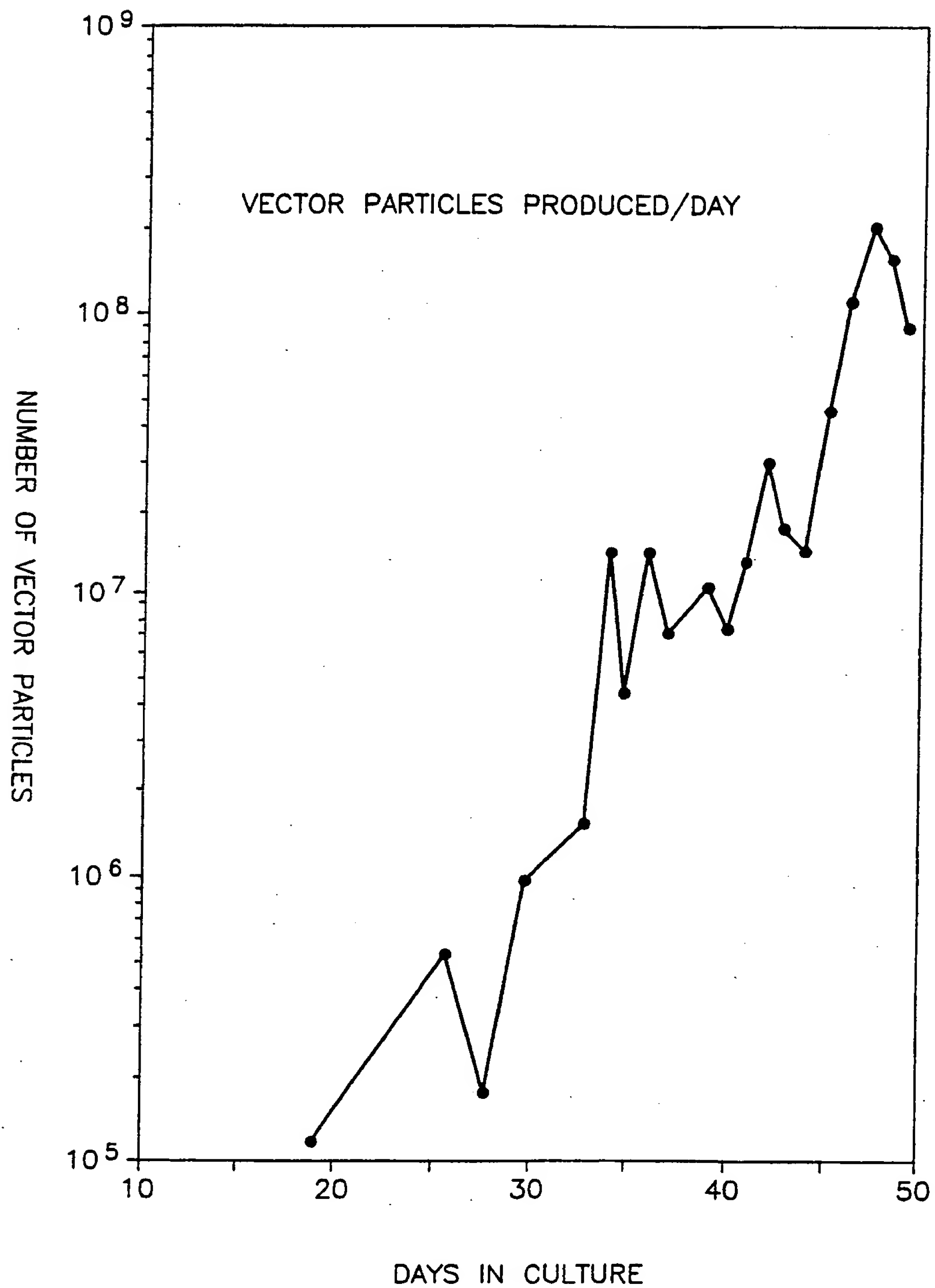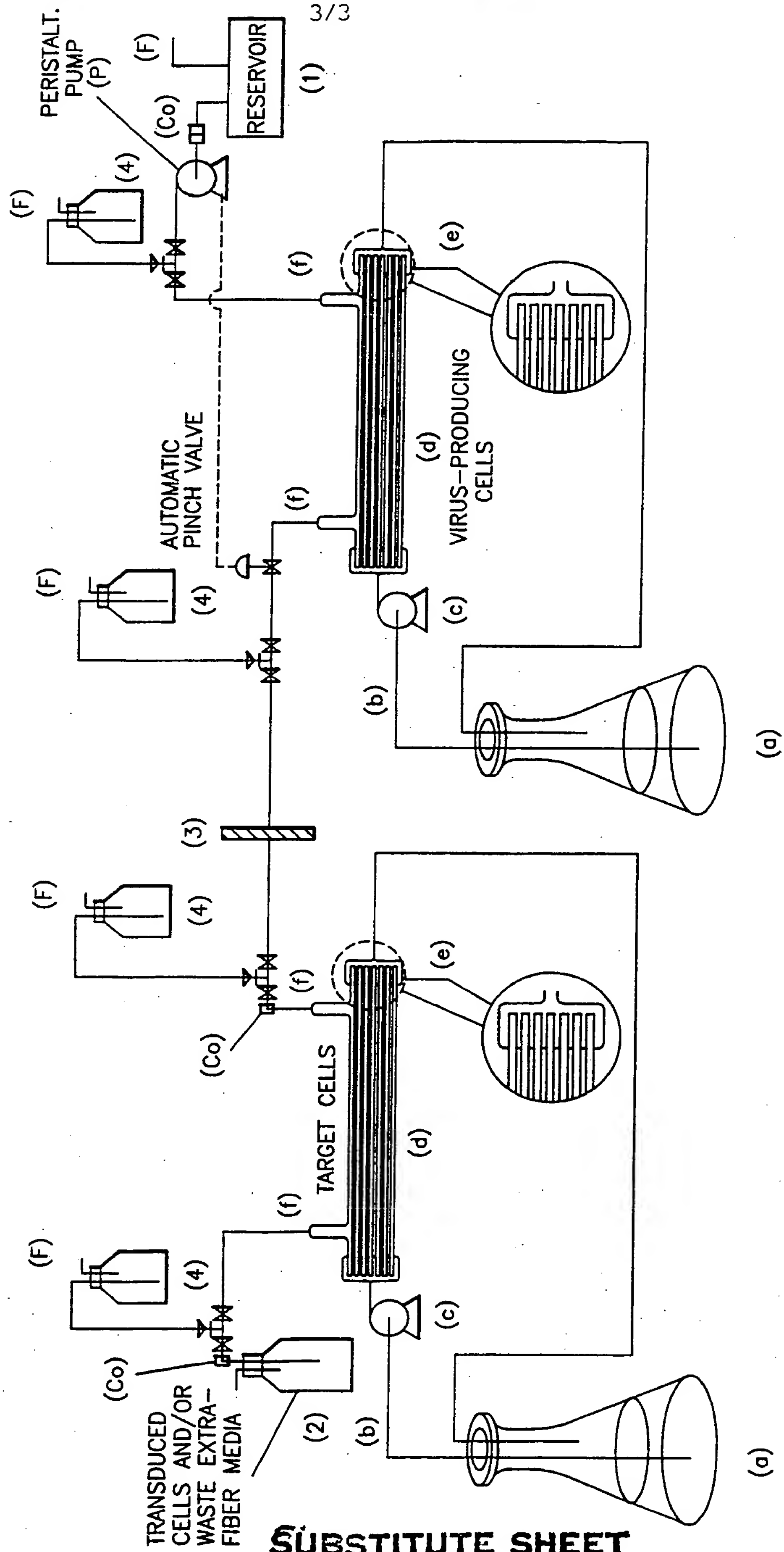
FIG. 1a

LASN RETROVIRAL VECTOR PRODUCTION

VECTOR PARTICLES/ml

NUMBER OF VECTOR PARTICLES

DAYS IN CULTURE

FIG. 1b

LASN RETROVIRAL VECTOR PRODUCTION

VECTOR PARTICLES PRODUCED/DAY

NUMBER OF VECTOR PARTICLES

DAYS IN CULTURE

BNSDOCID: <WO__9210564A1>

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FIG. 2

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 7/02, 5/00, 5/06; C12M 3/00
US CL : 435/239, 240.1, 240.242, 284

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/239, 240.1, 240.242, 284

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched[5]

DIALOG DATABASES: BIOSIS PREVIEWS 1985+, MEDLINE 1975+, NTIS, CA SEARCH,
BIOTECHNOLOGY ABSTRACTS 1982+

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category[*]	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,999,298 (Wolfe et al) 12 March 1991. See entire document.	1-52
Y	US, A, 4,861,719 (Miller) 29 August 1989. See entire document.	1-52
Y	US, A, 4,301,249 (Markus et al) 17 November 1981. See entire document.	1-52
Y	Enzyme Microb. Technol., Vol. 9, issued September 1987, M. A. Tyo et al, "Dense cultures of animal cells at the industrial scale," pages 514-520. See entire article.	1-52
Y	Poster presentation at Bio-Expo 86, issued 29 April 1986 (Boston MA), Tsang et al, "Production of HTLV-III Virus in Cells Grown on Artificial Capillaries," No. 510148-1. See entire article.	1-52

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
03 MARCH 1992	17 MAR 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Johnny F. Railey II

Form PCT/ISA/210 (second sheet) (May 1986) ß

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

   Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

   ☐ The additional search fees were accompanied by applicant's protest.

   ☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet(2))(Rev. 4-90) ß

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS
(Not for publication)

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-19, 35, 36 and 38, drawn to a first method, for producing a high titer of recombinant viral or retroviral vectors.

II. Claims 20-34, 37, 39-42, 51 and 52, drawn to a second method, for producing transduced target cells.

III. Claims 43 and 44, drawn to a first product, compositions of transduced target cells.

IV. Claims 45-47, drawn to a second product, transduced target cells produced by the methods of claims 20, 30 or 31.

V. Claim 48, drawn to a third product, a therapeutic composition of transduced target cells.

VI. Claims 49 and 50, drawn to a fourth product, a dual bioreactor perfusion circuit.